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The use of urocanic acid being able to acidify the cell cytoplasm and for preventing or halting cellular proliferation in a person.

FIELD OF THE INVENTION

This invention relates to the use of urocanic acid or another pharmacologically acceptable agent for acidifying cell cytoplasm and subsequently preventing or halting proliferation of cells, especially tumour or other transformed or non-transformed hyperproliferating cells in a person or an animal, and to treatment or prevention of cancer and hyperproliferative diseases curable by arresting cell growth and proliferation.

BACKGROUND OF THE INVENTION

The publications and other materials used herein to illuminate the background of the invention, and in particular, cases to provide additional details respecting the practice, are incorporated by reference.

Many cellular functions in both normal and transformed cells are linked to the maintenance of intracellular pH. Several investigators have recently shown that the proliferation activity of cancer cells can be modulated by agents that are capable of acidifying the cell cytosol (Cosentini *et al.* 2001, Wahl *et al.* 2002, Thangaraju *et al.* 1999). Analogously, intracellular acidification activates apoptosis or programmed cell death cascades (Gottlieb *et al.* 1996, Matsuyama *et al.* 2000). Acidification is postulated to affect key apoptotic enzymes such as acidic endonuclease causing DNA fragmentation and acidic sphingomyelinase producing ceramide (Gottlieb *et al.* 1996). Controlling of cell proliferation activity and apoptosis has consequently been identified as a promising approach for the pharmacological intervention in cancer (reviewed in Los *et al.* 2003). In the tumour bed, the cytosolic pH of the viable tumour cells is typically maintained close to neutral to facilitate proliferation, whereas the extracellular microenvironment is acidified by cellular metabolites (Yamagata & Tannock 1996).

SUMMARY OF THE INVENTION

The inventors of the present invention have demonstrated a so far unknown property of urocanic acid (UCA). They have surprisingly shown that UCA migrates into the cytosol of

malignant and non-malignant cells in a form which is able to release a proton in the cytosol. Subsequently, the cytoplasm is acidified (pH is decreased), and as a further result thereof, normal or abnormal cell proliferation activity is prevented.

Thus, according to one aspect, this invention relates to the use of UCA or another pharmacologically acceptable agent being able to acidify the cell cytoplasm, for the manufacture of a pharmaceutical composition useful for preventing or halting cellular proliferation in a person or an animal, wherein an effective amount of UCA or another agent is administered in an essentially non-dissociated form to the person or animal.

According to another aspect, this invention relates to the use of UCA or another pharmacologically acceptable agent as an enhancer for another therapeutically active agent.

According to a third aspect, the invention concerns a pharmaceutical composition of UCA or another pharmacologically acceptable agent being able to acidify the cell cytoplasm, in combination with a pharmaceutically acceptable carrier, which carrier essentially prevents the agent from dissociating at extracellular pH values.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the anti-proliferative effect of camptothecin on cell lines. The cells were cultured for 44 h with or without 2 μM camptothecin (CPT) at pH 6.5 or 7.4 conditions. After a 2-h incubation with the proliferation assay reagent, the absorbance at 490 nm was recorded. Cells were cultured in a density of 15,000 cells (K562, HT-1080, HK293) or 75,000 cells (the other cells) in a volume of 150 μl.

Figure 2 shows the anti-proliferative effect of *cis*-UCA in combination with camptothecin and low pH adaptation in two skin melanoma cells lines from humans. The cells were cultured in triplicate with or without 2 μM camptothecin (CPT) or 10 mM *cis*-UCA in a density of 15,000 cells per 150 μl for 92 h. *A*, Cells taken from normal pH 7.4 cultures to test conditions at pH 6.5. *B*, Cells cultured in low pH 6.7 adaptation medium for 3 d, then tested at pH 6.5. *C*, Cells cultured and tested at pH 7.4.

Figure 3 shows the concentration response of *cis*-UCA to A2058 melanoma cell proliferation. Proliferation was measured in a 44-h assay at pH 6.5 with 30 μM to 30 mM *cis*-UCA and 2 μM camptothecin. Both proliferation (*A*) and calculated percentage inhibition data (*B*) are presented.

Figure 4 shows the additive anti-proliferative effect of *cis*-UCA. Three cell lines were cultured with *cis*-UCA and/or 1 μM camptothecin at a density of 30,00 cells per well (100 μl) for 44 h in pH 6.5 medium. Blank wells with all the *cis*-UCA concentrations but without cells were included and their absorbance subtracted from those of the corresponding test wells.

Figure 5 shows the intracellular pH measurement in UCA-treated tumour cells in situ. The cells were labelled with BCECF and placed in pH-adjusted buffer solutions with or without various concentrations of *cis*-UCA. *Left panels*, calibration of BCECF fluorescence intensity of nigericin-treated cells in high-potassium buffer as a function of buffer pH. *Right panels*, intracellular pH values in buffers with or without various concentrations of *cis*-UCA. All test buffers were adjusted to pH 7.4 (%) or pH 6.5 (+) after the addition of *cis*-UCA. Results were calculated using the corresponding calibration curve on the left.

DETAILED DESCRIPTION OF THE INVENTION

According to a preferable embodiment, the pharmaceutically acceptable compound is urocanic acid (UCA), but it is not restricted hereto. Any other pharmaceutically acceptable, non-toxic acid or base having its dissociation constant in the range 5.0 to 7.4, preferably in the range 6.0 to 7.3; most preferably about 7.0, and being able to accumulate inside a cell would be useful. Such compounds may be inorganic or organic, preferably an organic agent having, like UCA, an heterocyclic ring to which a saturated, or more preferably, an unsaturated carboxylic acid moiety is attached. The heterocyclic group may be, for example, an imidazole (as for UCA) or any other heterocyclic or poly-heterocyclic group having the ability to donate a proton at cytoplasmic pH and thereby acidify the cytoplasm. As examples of other suitable heterocyclic groups can be mentioned thiazole, thiophene, furan, oxazole, triazole, tetrazole, pyrazole, pyridine, pyrimidine and triazine.

The pharmaceutically acceptable compound is admixed with a carrier, which can be one single component, or more preferably, a mixture of two or more components. One of the components is suitably a buffering agent, which adjusts the pH of the composition to the desired value. Especially when the *trans*-isomer of UCA, *trans*-UCA, is the active agent, it is preferable to adjust the pH of the composition to 4.0 to 6.1, more preferably 5.0 to 6.1. In this pH range, *trans*-UCA is still non-dissociated. When the *cis*-isomer of UCA, *cis*-UCA, is the active agent, it is preferable to adjust the pH of the composition to 5.0 to 7.0, more preferably 6.0 to 7.0. In this pH range, *cis*-UCA is still non-dissociated.

According to one embodiment, the pharmaceutical composition can also comprise another therapeutically active agent, the effect of which is enhanced by UCA. Preferably, such a therapeutically active agent is, but not limited to, an anti-proliferative or an anticancer drug.

As examples of suitable buffering agents to adjust pH to 4.0-7.0 can be mentioned, but not restricted hereto, 50 mM sodium phosphate supplemented with 55 mM sodium chloride, cell culture media with 25 mM HEPES, and 10 mM Pipes supplemented with 133 mM sodium chloride.

The method and composition according to this invention is useful for treatment or prevention of cancer and hyperproliferative diseases curable by intracellular acidification. The term intracellular acidification used herein refers to the elevation of hydrogen ion concentration in the cytosolic or subcellular compartments in a eukaryotic cell.

Hyperproliferative conditions that can be treated or prevented according to the present invention are any forms of cancer, such as, but not limited to, brain, skin (such as melanoma), bladder, gastric, pancreatic, breast, head, neck, oesophageal, prostate, colorectal, lung, renal, gynaecological (such as ovarian) or thyroid cancer; other epitheliomas; cysts in various organs; warts and wart-like tumours induced by virus infection; fibrosarcoma and its metastases. In another embodiment, the present invention relates to treatment of non-cancerous hyperproliferative disorder, such as benign hyperplasia of skin or prostate (e.g. benign prostatic hypertrophy), synovial hyperplasia in rheumatoid arthritis, inflammatory bowel disease, restenosis, atherosclerosis, thrombosis,

scleroderma or fibrosis. Most preferably the target cells of the method and compositions of the present invention are cells comprising solid tumours in the skin.

For the purpose of this invention, the pharmaceutically acceptable agent can be administered by various routes, either systemically or locally. The suitable administration forms include, for example, cutaneous formulations; intratumoural injections including intravenous, intramuscular, intradermal and subcutaneous injections; intrasynovial injections; and mucosal, topical, transdermal, nasal, inhalation or rectal formulations. Particularly suitable formulations are formulations for local delivery such as topical formulations in the form of ointments, gels, creams, pastes, solutions, suspensions, lotions and emulsions. Also targeted drug delivery systems, such as liposomes and nanoparticles, in combination with aforementioned administration forms can be used for administration of the pharmaceutically acceptable agent.

The required dosage of the pharmaceutically acceptable compound will vary with the particular condition being treated, the severity of the condition, the duration of the treatment, the administration route and the specific compound being employed. In a topical formulation the amount of the pharmaceutically acceptable compound can typically range from 0.01 % to 50 %, preferably in the range 0.1 to 10 %.

The invention will be illuminated by the following non-restrictive Experimental Section.

EXPERIMENTAL SECTION

The aim of the present study was to investigate the hypothesis that UCA enters into transformed cell types in an appropriate extracellular environment, acidifies their cytoplasm and subsequently inhibits the proliferation of the cells.

In a previous publication (WO 2004/080456), we have demonstrated that both *cis*-UCA and *trans*-UCA rapidly and irreversibly accumulate into the cytosol in live human polymorphonuclear neutrophils. There was also no indication that UCA would bind to intracellular organelles nor that it would be metabolised in the cytosol. The *cis*-UCA but not *trans*-UCA affected the intracellular pH of neutrophils when the extracellular pH was in a pH range of 6.1 to 7.0. In these conditions the *cis*-UCA is still non-dissociated whereas

trans-UCA, with the pK_a of 6.1, is mostly or completely dissociated and thereby was not able to transport protons in the cytosol. However, when the extracellular pH is below 6.1, e.g. in a range of 5.0 to 6.0, also *trans*-UCA is able to act as proton carrier and it induces intracellular acidification. For the sake of clarity, the experimental results here show only the effects of *cis*-UCA, although similar results can be obtained with *trans*-UCA in an appropriate pH range.

Intracellular acidification is known to stand as a prerequisite for changes in many cell functions such as growth arrest. It is also known that the growth (proliferation) of transformed cell types such as cancer cell lines can be inhibited by agents that are able to cause intracellular acidification. The usage of such compounds (usually organic acids) in clinical treatment of cancer may be hindered by their adverse side effects or toxicity.

Methods

Urocanic acid

Trans-urocanic acid [*trans*-UCA, 3-(1*H*-imidazol-4-yl)-2-propenoic acid, MW 138.14] was purchased from Sigma (St. Louis, MO, USA). *Cis*-UCA was prepared from *trans*-UCA with UV photoisomerisation as follows. *Trans*-UCA (138 mg, 1 mmol) was dissolved in water (500 ml). The solution was brought to pH 9 with solid potassium hydroxide and then irradiated under nitrogen atmosphere at 10 °C for 4 h. Photoisomerisation was performed in a Normag falling-film photoreactor with Hanau quartz mercury high-pressure lamp (500 W, 270–350 nm). The resulting mixture (*trans*/*cis* *ca.* 30/70 by HPLC) was evaporated to dryness and the residue dissolved in 12.5 mM acetic acid. This solution was adjusted to pH 9 and chromatographed on an ion exchange column (25 x 2.3 cm, 200–400 mesh, acetate form, Bio-Rad 1-x8) using 12.5 mM (500 ml), 25 mM (500 ml), and 100 mM (1000 ml) acetic acid as successive eluents. *Cis*-UCA appeared after *ca.* 1100 ml and *trans*-UCA mainly after 1300 ml eluent volumes. Removal of the solvent from the fractions, followed by washing with diethyl ether and drying *in vacuo* at 65 °C over phosphorus pentoxide, yielded the pure *trans*- and *cis*-isomers. The yield of *cis*-UCA was 85 mg (58 %), mp. 176–178 °C, with more than 99.5 % chemical purity by HPLC analysis. An aminopropyl stationary phase column Lichrosorb NH_2, Hibar RT, 250 x 4 mm, 5 μm (Merck, Darmstadt, Germany) was used for HPLC analysis. The

eluent was a 50 % (v/v) mixture of acetonitrile and a solution of 2 % (v/v) acetic acid and 0.5 % (w/v) ammonium acetate in water (pH *ca.* 5). The isomers were detected at 268 nm, and the retention times were T_r(*cis*) 3.7 min and T_r(*trans*) 5.4 min.

Cis-UCA was dissolved directly in the incubation buffer or culture medium up to 30 mM concentration, sterile filtered (0.2 µm), and diluted into the desired concentrations immediately before the start of each experiment.

Camptothecin

Camptothecin (Sigma, MW 348.4) stock solution was prepared by dissolving the reagent (1.4 to 2.1 mg/ml) in deionised water. The solution was alkalinised with 1N NaOH, and dissolution was further aided with heating in a boiling water bath. Dilutions of the stock solution were made in physiological saline. Final concentrations in the experiments were 20 to 2000 nmol/l.

Cell lines

Transformed tumour cell lines WM 266-4 and A2058 (cutaneous melanoma), HT-1080 (epithelial fibrosarcoma), HeLa (epithelial adenocarcinoma of cervix), HK293 (kidney epithelial cells) and non-transformed cell line HSF (skin fibroblast from a healthy male volunteer donor) of human origin have been described earlier (Li *et al.* 2003). The K562 (chronic myelogenous leukemia) cells were purchased from American Type Culture Collection.

The cells were maintained in logarithmic growth phase in IMDM medium (Invitrogen, Paisley, UK) supplemented with 10 % fetal calf serum and antibiotics in a humidified incubator at +37 °C, 5 % CO_2. The adherent cells lines were harvested for experiments with 0.25 % trypsin-EDTA in PBS for 5 min and then resuspended and washed in medium. After counting of viable cells, 15,000 to 75,000 cells were transferred into flat-bottom 96-well cell culture plates in a volume of 100 or 150 µl.

Proliferation assay

The proliferation activity of cultured cell lines was quantified in flat-bottom 96-well plates with a modified colorimetric assay based on a tetrazolium derivative (CellTiter 96 Aqueous One Solution Cell Proliferation Assay, Promega). The cells were cultured in the presence of camptothecin and/or *cis*-UCA for 20 to 92 h, then the proliferation reagent was added for 2 h, and the absorbance at 490 nm was measured in a plate reader. Blank absorption values in wells containing medium without cells were subtracted before comparison analysis.

Monitoring of intracellular pH

Intracellular pH in the cell lines in the presence of *cis*-UCA was measured with the pH-sensitive fluorescent dye 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF, acetoxymethyl ester; Molecular Probes, Leiden, The Netherlands) by flow cytometry. Two million cells were incubated in 5 ml DMEM medium (Invitrogen), pH 7.4, containing 0.35 μM BCECF at 37 °C for 30 min, washed once in sodium phosphate buffer (50 mM NaH_2PO_4/Na_2HPO_4, 43 mM NaCl), pH 7.4, and resuspended in 0.3 ml saline. Twenty μl of the cell suspension was pipetted into flow cytometry tubes. Sodium phosphate buffer solutions with or without *cis*-UCA were adjusted (with 0.1 N HCl/NaOH) to pH 6.5 or 7.4 after the addition of *cis*-UCA and added into the tubes to yield a final volume of 500 μl. Flow cytometry analysis was performed within one hour.

Calibration of intracellular pH in situ was performed using the K^+/H^+ ionophore nigericin (Molecular Probes) in high-potassium buffers. BCECF-labelled cells (20 μl in saline) were resuspended in pH-adjusted calibration buffers (480 μl of 50 mM KH_2PO_4/K_2HPO_4, 43 mM KCl, pH 6.2, 6.5, 6.8, 7.2, and 7.5) in flow cytometry tubes. Nigericin stock solution (10 mM in methanol) was diluted 1:10 in saline and 5 μl added to the calibration cell suspension shortly (10 to 15 min) before analysis. The cells were kept at room temperature during the experiment. Exact intracellular pH values were calculated from mean BCECF fluorescence intensities by reference with nigericin-clamped calibration cells. Calibration was performed separately and simultaneously for each cell line.

Results

Camptothecin shows anti-proliferative action that is enhanced at lower pH

The base line proliferation in most cell lines was higher at pH 7.4 cultures than at pH 6.5. Camptothecin (2 μM) inhibited the proliferation in all cell line cultures and showed more efficient inhibition at pH 6.5 **(Fig. 1)**.

Cis-UCA inhibits cancer cell proliferation and augments the anti-proliferative action of camptothecin at pH 6.5

Based on our previous observations with peripheral blood neutrophils that *cis*-UCA exerts inhibitory effects in its non-dissociated molecular form, i.e. in the extracellular pH range 6.1 to 7.0, we investigated *cis*-UCA action in the melanoma cell lines A2058 and WM266-4 at two pH levels, pH 6.5 and pH 7.4. The 10 mM *cis*-UCA significantly inhibited cell proliferation both by itself and it also enhanced the inhibition by camptothecin when the normally (at pH 7.4) cultured cells were tested at pH 6.5 culture medium **(Fig. 2A)**. The percentage inhibition by 10 mM *cis*-UCA alone was 83 % (p=0.0028) in A2058 cells and 36 % (p=0.0020) in WM266-4 cells. The proliferation of cells first adapted to pH 6.7 culture conditions for 3 days before the beginning of the test culture period at pH 6.5 was in the same order (for *cis*-UCA, 87 %, p=0.0056 and 28 %, p= 0.090, respectively) **(Fig. 2B)**. Inhibition at pH 7.4 was 2 to 3 % only **(Fig. 2C)**. Similarly, the effect of camptothecin was again better at the lower pH (Fig. 2). These experiments showed that *cis*-UCA acts on tumour cells as an anti-proliferative agent when the extracellular pH < pK_a or when the imidazolyl moiety of the molecule is in the non-dissociated form.

The cell lines were tested in a 44-h culture at pH 6.5 with or without 2 μM camptothecin and 10 mM *cis*-UCA at 15,000 cells per 100 μl total volume. In these conditions, *cis*-UCA alone significantly inhibited cell proliferation (by 20 % to 50 %) and enhanced the inhibitory effect of camptothecin in most cell lines **(Table I)**. In order to further characterise the effective anti-proliferative concentration range of *cis*-UCA, proliferation was measured in a 44-h assay at pH 6.5 with *cis*-UCA concentration ranging from 30 μM to 30 mM. The results for A2058 melanoma cells show that *cis*-UCA is required in at least 3 mM concentration to produce measurable inhibition **(Fig. 3)**.

Table I Inhibition of proliferation by *cis*-UCA (10 mM) and camptothecin (CPT, 2 μM) in cell lines at pH 6.5.

Cell line[1]	CPT	*cis*-UCA	CPT + *cis*-UCA
HSF	37 ± 0.4 %[2]	41 ± 1.1 %	69 ± 2.3 %
	p=0.00056	p=0.0053	p=0.00020
WM266-4	81 ± 1.1 %	20 ± 0.6 %	80 ± 3.4 %
	p=0.0016	p=0.044	p=0.0012
A2058	84 ± 3.3 %	45 ± 4.4 %	89 ± 1.0 %
	p=0.0069	p=0.038	p=0.0054
HK293	56 ± 1.0 %	22 ± 0.1 %	55 ± 3.3 %
	p=0.0035	p=0.013	p=0.00080
HeLa	78 ± 1.6 %	50 ± 1.7 %	85 ± 1.7 %
	p=0.00046	p=0.0032	p=0.00042
K562	77 ± 3.3 %	26 ± 2.9 %	79 ± 18 %
	p=0.0018	p=0.016	p=0.0069
HT-1080	71 ± 1.7 %	47 ± 0.5 %	79 ± 4.1 %
	p=0.00035	p=0.0024	p=0.0018

[1]15,000 cells per 100 μl were cultured in triplicate wells for 44 h.

[2]Mean percentage inhibition ± SD compared with untreated control.

In a further experiment, a lower camptothecin concentration (1 μM) was used to characterize the additive anti-proliferative effect of *cis*-UCA. In all three studied transformed cell lines, *cis*-UCA inhibited proliferation at 3 mM or higher concentration **(Fig. 4)**. The additive effect of *cis*-UCA to camptothecin was evident in the 1 to 10 mM range depending on cell line. At the largest *cis*-UCA concentration studied (30 mM),

proliferation with *cis*-UCA alone was always at the same level than in combination with camptothecin and lower than with 1 μM camptothecin alone (Fig. 4).

Cis-UCA decreases intracellular pH in transformed cells

Because *cis*-UCA showed anti-proliferative effects in the cell lines, we next investigated whether *cis*-UCA produces intracellular acidification in these cells. We have previously shown that *cis*-UCA accumulates in high concentrations in the cytosol of peripheral blood neutrophils and lowers the pH of the cytosol when the extracellular pH is adjusted below 7.0. Selected transformed cell lines were labeled with pH-sensitive fluorescent dye BCECF and placed in pH-adjusted buffer solutions with or without various concentrations of *cis*-UCA. Flow cytometer analysis showed that the intracellular pH in A2058 and HeLa cells remained almost constant with escalating *cis*-UCA concentrations up to 30 mM when the extracellular pH was kept at 7.4. When the extracellular buffer was adjusted to pH 6.5, however, the intracellular pH decreased in *cis*-UCA concentration-dependent fashion in the range 0.3 to 30 mM. The decrease was about 0.25 pH units with the highest *cis*-UCA concentration **(Fig. 5)**. These data with A2058 and HeLa cells show that *cis*-UCA is able to acidify the cytosol of transformed cells. The observed acidification is likely to be an initiating event in the anti-proliferative effect of *cis*-UCA.

Conclusions

Tumour cells acquire their capacity to escape the tight regulation of cell division through transformation. In this multi-step process, inactivation of intracellular pathways that restrain proliferation and activation of those that promote it are key events. The resulting abnormal growth behaviour of transformed cells remains a major challenge in medicine. Because cancer cells do not generally obey the normal cellular mechanisms of cell cycle progression and apoptosis, and solid tumours create an abnormal acidic and hypoxic microenvironment, the efficacy of drug therapies is often compromised, but may also offer a novel perspective for the design of tumour-selective drugs (Kozin *et al.* 2001). The cells of solid tumour tissue tend to maintain a pH gradient across the cell membrane; the cytosolic pH is close to neutral, whereas the extracellular microenvironment is acidic, usually around pH 6.7 (Kozin *et al.* 2001, Yamagata & Tannock 1996). At low extracellular pH, the uptake of certain drugs like camptothecin into cells is enhanced (Gabr

et al. 1997), and acidic intracellular environment is required for efficient anti-proliferative activity because of reversible transformations in the molecule structure (Burke & Mi 1993). It has been shown that the chemosensitivity of tumour cells to camptothecin can be enhanced by the simultaneous treatment with acids that are capable of acidifying the intracellular environment (Cosentini *et al.* 2001, Gabr *et al.* 1997). Camptothecin itself acidifies cytosol of leukaemia cells in a time frame of several hours, leading to induction of apoptosis (Goossens *et al.* 2000). Intracellular acidification by a complementary proton carrier could enhance the effect of camptothecin and corresponding drugs. Similarly, the interaction of alkylating and platinum-containing drugs with DNA prefer low intracellular pH milieu (Jahde *et al.* 1989, Atema *et al.* 1993)

The effective concentrations of *cis*-UCA used in the present experiments are in the millimolar scale, mostly between 1 and 30 mM. The natural location of *cis*-UCA is the surface layer of the epidermis where the published concentrations of total UCA are in the range 0.5 to 8.9 mM, considering the mean thickness of the epidermis (Laihia *et al.* 1998). It is therefore possible that these concentrations are important in maintaining an innate antibacterial, anti-proliferative, and anti-inflammatory acidic surface environment ("acid mantle", Ohman & Vahlquist 1994) against pathogenic micro-organisms, tumours, and neutrophil accumulation, respectively. A recent analysis provides evidence that UCA is the major effector molecule in the acid-base homeostasis in the epidermis (Krien & Kermici 2000). On the other hand, the concentrations of other organic acids experimentally augmenting camptothecin anti-proliferative activity lie in the same range. As an example, the histone deacetylase inhibitors phenylbutyrate and phenylacetate reduced the proliferation of colon carcinoma cells *in vitro* by 20 % to 86 % in the concentration range 5 to 40 mM, respectively (Cosentini *et al.* 2001).

Although not yet studied in detail, the characteristics of UCA as a potential pharmacological substance in cancer intervention are promising. First, UCA is a natural molecule that is synthesised in large quantities in the skin, as explained above. UCA has been known for more than 120 years and is the topic of hundreds of biomedical publications. These facts exclude suspects on surprising pharmacological toxicity or possibility to allergic sensitisation. Second, *cis*-UCA is water-soluble and penetrates readily into tissues and cell cytoplasm. Third, there is no known catabolism of *cis*-UCA in mammals, and thus no adverse effects of possible metabolites can be expected. In the skin

in vivo, *trans*-UCA is synthesised from histidine (Baden & Pathak 1967). The UV-induced photoisomerisation produces the *cis* isomer that is then excreted as such in the sweat and in urine. If unintentional photoisomerisation back to *trans*-UCA should take place after *cis*-UCA medication, systemic *trans*-UCA is metabolised via multiple harmless intermediates to glutamate in a natural way in the liver. No endogenous metabolism in the skin exists. Fourth, the proton dissociating properties of *cis*-UCA make it potential cancer drug. UCA is a weak polyprotic acid, with two proton-donor moieties, the carboxyl group and the imidazolyl group. The first pK_a referring to the carboxyl group of *cis*-UCA is 3.3 (Roberts *et al.* 1982). Practically all *cis*-UCA molecules are therefore deprotonated at the carboxyl group at pH > 4. At the physiologically relevant pH levels, the protonation status of the imidazolyl group alone determines whether the molecule is able to donate a proton and thereby promote acidification. The second pK_a, that of the imidazolyl group, of *cis*-UCA is 7.0 (Roberts *et al.* 1982, Krien & Kermici 2000). Consequently, the imidazolyl group of *cis*-UCA is protonated at pH < 7.0 only and can act as a proton donor when entering the cytoplasm which has pH > 7.0. It has been noted that the acidification-induced apoptosis in cancer cells is the most efficient when the intracellular pH is close to 6.5 (Thangaraju *et al.* 1999). Activation of caspases by mitochondrial cytochrome C is pH-sensitive with an optimum in the pH range 6.3 to 6.8 *in vitro* (Matsuyama *et al.* 2000). The data presented here indicate that *cis*-UCA has potent anti-proliferative activity in viable cancer cells at an extracellular pH 6.5 but limited activity at pH 7.4. The proton dissociation properties of cancer drugs has been discussed, but on the point of view of cellular uptake only (Kozin *et al.* 2001). In addition to good uptake characteristics, an optimal drug for acidifying cells in tumours would have a pK_a value higher than the actual extracellular pH and lower than the intracellular steady state pH. In the mildly acidic extracellular conditions, tumour cell proliferation should be seized by *cis*-UCA-induced intracellular acidification. This idea may also apply to other conditions of hyperproliferation, such as proliferation of synovial fibroblasts in rheumatoid arthritis. In the context of pharmacological treatment of solid tumours or other possible hyperproliferating cells, mildly acidic extracellular conditions may be ideal for the use of *cis*-UCA.

In conclusion, the present study shows data which, for the first time, surprisingly demonstrates the anti-proliferative action of UCA on cancer cells. The modulation is related to the property of UCA to acidify the cell cytosol. The acidification property is based on a favourable pK_a of UCA and is thus restricted to the condition where the

cytosolic pH is close to or beyond neutral and the extracellular environment is mildly acidic. These pH conditions generally prevail in solid tumour tissue.

The invention is further illuminated by the following non-restricting Examples.

EXAMPLES OF FORMULATIONS ACCORDING TO THE INVENTION

Gel Composition 1 (% w/w)

Cis-urocanic acid		0.1-10
Carbopol 974		1.5
Propylene glycol		12.5
Buffering agent		0.01-1
Purified water	to	100

Gel Composition 2 (% w/w)

Cis-urocanic acid		0.1-10
Natrosol (hydroxyethyl cellulose)		1.0
Buffering agent		0.01-1
Purified water	to	100

Cream Composition 1 (% w/w)

Cis-urocanic acid		0.1-10
Propylene glycol		50
Cetostearyl alcohol		15
Sodium lauryl sulphate		1
Buffering agent		0.01-1
Purified water	to	100

Cream Composition 2 (% w/w)

Cis-urocanic acid		0.1-10
Cetostearyl alcohol		6.75
Propylene glycol		40
Sodium lauryl sulphate		0.75
Poloxamer 407		1
Mineral oil		5
Stringy petrolatum		12.5
Buffering agent		0.01-1
Purified water	to	100

Ointment Composition (% w/w)

Cis-urocanic acid		0.1-10
Mineral oil		5
Buffering agent		0.01-1
Petrolatum	to	100

It will be appreciated that the methods of the present invention can be incorporated in the form of a variety of embodiments, only a few of which are disclosed herein. It will be apparent for the expert skilled in the field that other embodiments exist and do not depart from the spirit of the invention. Thus, the described embodiments are illustrative and should not be construed as restrictive.

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